Encapsulation of fish oil using H_2O_2-oxidative modified sodium alginate and carrageenan mixture to reduce oil leakage

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Declaration

I certify that this thesis does not contain material, which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis or in the notes.

Dhrub Ghimire

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Abbreviations

Sod. alginate	Sodium alginate
kDa	Kilodaltons
G/M	Guluronate/Mannuronate
GA	Guluronic Acid
MA	Mannuronic Acid
Mw	Molecular weight
h	Hour
min	Minute
Alg	Alginate
Carr.	Carrageenan
mPas	Millipascal second
N/m	Newton/meter
EE	Encapsulation efficiency
STDEV	Standard deviation
IPN	Inter-penetrating polymer network
HPLC	High performance liquid chromatography

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Abstract

Alginate and carrageenan are commonly identified polysaccharides that are contained in the seaweed (brown algae) and red algae respectively. These are the polysaccharides with high commercial value due to their gelling and stabilizing properties. These polysaccharides have shown various commercial application individually and in incorporation with one another. Alginates have molecular weight in ranges from low to high (commercially available alginates ranging between 32 kDa and 400 kDa). Their gelling property with ions like calcium is dependent on the molecular weight and viscosity. The first phase this experiment, made modifications on the molecular weight and viscosity of the sodium alginate (Sigma Aldrich) using hydrogen peroxide as an oxidizing/depolymerizing agent at different time conditions (30 min to 3h) and concentration of hydrogen peroxide (0.3% v/v, 0.5% v/v and 1.0% v/v). Then, determined the molecular weight and viscosity of the oxidized alginate at different oxidation time and concentration of hydrogen peroxide used using HPLC and viscometer. The viscosity and MW of oxidized were significantly reduced, the lowest being at 3h of oxidation. Also, the oxidation process was rapid during the first hour when 1.0% (v/v) of the hydrogen peroxide was used for oxidation. And the second phase of the experiment, studied the gelling behavior of the oxidized alginate whether it has same gelling property as before. Also, G/M (Guluronic acid and Manuronic acid) ratio of the oxidized alginate were determined to study if there is any change in the G/M ratio. Guluronate (G) part of the alginate is responsible for the gelling property and there was no significant difference between the control (unoxidized from Sigma Aldrich) and oxidized alginate. This gave the idea that the modified alginate still has good gelling property. Next, we incorporated this modified alginate with carrageenan to make a strong and stable biofilm that can be used for commercial applications. Alginate and carrageenan were mixed in different ratios making final concentration of 1% (w/v) and the mixture solution was allowed to form gel in 0.5% (w/v) calcium chloride containing 20% (v/v)

ethanol and physical properties were studied. The film made up of combination of 0.3% (w/v) alginate and 0.7% of carrageenan (w/v) was selected for the study of the stress, strain and swelling ratios. The mixed film showed slow and steady swelling behavior compared to only alginate and only carrageenan gels. Finally, we used 0.3% (w/v) oxidized alginate and 0.7% (w/v) carrageenan mixture to encapsulate the fish oil (10% v/v) and the encapsulation efficiency and stability of the encapsulated beads were compared with final 1% w/v (0.3% w/v medium viscosity alginate and 0.7% w/v carrageenan mixture w/v). The modified alginate-carrageenan mixture showed excellent efficiency and very good stability after 1 week of incubation than the 1% (w/v) unmodified alginate-carrageenan mixture. We used amount of beads surface oil and oil in gelling solution to determine the efficiency of encapsulation and stability. Most stable were the beads lower the surface oil of the encapsulated beads. This experiment found that higher molecular weight sodium alginate can be oxidized to make low molecular weight and less viscous without losing its gelling properties and the modified alginate can be incorporated with another polysaccharide carrageenan to form a more stable material that can have high commercial application (encapsulation of fish oil done in this experiment). High molecular weight and high viscosity alginate are found to be immunogenic and hard to biodegrade, so this study can significantly address that problem. However, there are some limitation of this study as we used only two different types of alginate (unoxidized and oxidized) and carrageenan. In further research polysaccharides with varying molecular weight and viscosity can be studied. Also, the properties of mixed polysaccharide films can be optimized for commercial application that can be used as packaging material and that somehow could be the alternative to non-degradable commercial polyethene.

Key words: Alginate, carrageenan, encapsulation, stability, efficiency, G/M ratio

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Marine biotechnology could provide new understandings into the basic biological principles that will further develop various industries (Rayappa and Diwan, 2017). Today, there is a new interest in finding the marine habitat for new products that can be used in pharmaceutical and food industries (Romano et al., 2017). Marine microorganisms like bacteria, fungi, and microalgae today have come into the focus of the research community that were previously thought to be trivial, as they are found to be the sources of biologically active and commercially valuable compounds (Davidson, 1995). Various types and species of marine microalgae (seaweeds) like brown seaweed, red seaweed etc. are being studies to discover and identify the valuable biologically active compounds that can be used in various pharmaceutical and food industries. Seaweeds contain 80-90% water and 50% carbohydrate of their dry weight. Brown seaweed have 3-15% protein of their dry weight and red or green seaweeds have 10-47%. Similarly, they both contain 1-3% mineral of their dry weight (Khalil et al., 2017).

Seaweed hydrocolloids are of great significance (Li and Nie, 2016). Hydrocolloids are the substances that can form gel in the presence of water and are among the most commonly used ingredients in the food and pharmaceutical industries. They can be used as thickeners, gelling agents, emulsifiers, stabilizers clarifying agents (Qin, 2018). Beside this, they are recently found to have increasing use in the health products as they provide low calorie dietary fiber (Li and Nie, 2016). Hydrocolloid from seaweeds have potential future market and the market is growing due to their commercial applications like gelling agents and natural food thickeners (Bixler and Porse, 2011) as these compounds can enhance the food taste and stability of foods. The higher value of these products is due to higher price of seaweeds; specially like alginate and carrageenan and their shortages (Bixler and Porse, 2011).

The Philippines and Indonesia have mainly dominated the production of the carrageenan and carrageenan containing seaweeds, however these countries are also facing the factors that limits the production of seaweeds. Similarly, production in China have also been moving strongly however there is still lack of skills and values that can compete in the existing global market (Campbell and Hotchkiss, 2017).

Table 1.1 : Species and locations of hydrocolloid-producing seaweeds (Alba and Kontogiorgos, 2015)**.**

Species	***Occurrence***
Carrageenophytes *Kappaphycus alvarezii, Eucheuma denticulatum Indonesia, Philippines Gigartina skottsbergi, Sarcothalia crispate*	Indonesia, Philippines, Central Chile, Argentina
Agarophytes *Gelidium* *Gracilaria*	Spain, Portugal, Morocco, France, Japan, Republic of Korea, Mexico, Sumatra, Indonesia, lower harvests in Chile, China and South Africa Chile, Indonesia, Argentina, Atlantic coast of Canada, China, Vietnam, Namibia
Alginophytes *Ascophyllum nodosum, Laminaria digitate, Laminaria hyperborean, Macrocystis pyrifera, Laminaria japonica*	Ireland, Scotland, Norway France, Norway, Iceland Ireland, Scotland, Norway USA, Mexico, Chile

Table 1.2: Seaweed hydrocolloid sales volume: (Bixler and Porse, 2011)

Seaweed Hydrocolloid	***1999 sales (t)***	***2009 sales (t)***
Agar	7,500	9600
Alginates	23,000	26,500
Carrageenan	42,000	50,000
Total	72,500	86,100

Table 1.3: Seaweed hydrocolloids sales volume, latest updates (Porse and Rudolph, 2017)

Seaweed Hydrocolloid	***2009 Sales (t)***	***2015 sales (t)***
Agar	9,600	14,500
Alginates	26,500	24,600
Carrageenan	50,000	57,500
Total	86,100	93,035

From the above tables we can conclude that there is significant growth in seaweed hydrocolloids sales in last two decades.

Table 1.4: Seaweed hydrocolloids sales value (Porse and Rudolph, 2017).

Seaweed hydrocolloid	**2009 sales (Millions USD)**	**2015 sales (Millions USD)**	**% Change**
Agar	173	246	6
Alginates	318	345	8
Carrageenan	527	518	-1
Total	1018	1058	2

1.2. Alginates

The commonly identified polysaccharide that is contained in the seaweed (brown algae) is alginate, a glycuronan that has significant commercial value and have valuable to its gelling and stabilizing. It was first isolated in 1881 as '*algin*' from kelp (*Laminaria sp.*) by E.C.C Stanford (McHugh, 2003). Brown algae made polysaccharides up to 14-40% of their dry weight (Wells et al., 2017). These have been used in medical devices as entrapment beads in transplantation of compounds like insulin producing cells (Draget and Taylor, 2011). It is a collective term for the family of linear (1 - 4)-linked a-L-guluronan-D-mannuronans of widely varying composition and structure. This compound is found on the cell wall of the algae holding the cells together and that gives some structural and mechanical properties to the algae. In natural state, alginate is insoluble mixture of the salts that are prevalent in the marine environment like sodium, magnesium and calcium and remains in the swift ion-exchange balance with the sea water. Structural studies of alginates show that there are two different uronic acid residues as blocks of either D-manuronic acid residue (M- blocks) or L-glulуronic acid residue (G-blocks), separated by long chain of heteropolymeric material (MG-blocks) arranged in alternating way (Larsen et al., 2003).

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Fig 1.1: Alginate structural arrangement. (Larsen et al., 2003)

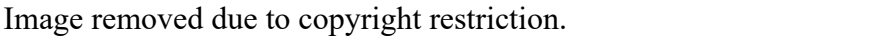

Fig 1.2: Representative alginate structure (Larsen et al., 2003)

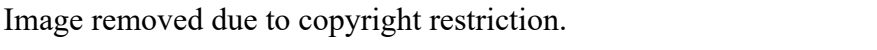

Fig 1.3: Difference between GGGG, MMMM and GMGM structures (George and Abraham, 2006)

The G-blocks of alginate are involved in intermolecular linking with the cations like calcium and form hydrogels. The M/G ratio, sequence and length of G-block are thus key factors that affect the physical properties of the alginate and corresponding hydrogels (George and Abraham, 2006). The mechanical properties of the alginate can be improved by increasing the G-block length and molecular weight affects its physical property and controls the stability of the gels (Aarstad et al., 2017).

Commercially available sodium alginates have molecular weight ranging between 32 kDa and 400 kDa. The viscosity of the alginate solution increases as pH is lowered and achieves maximum viscosity (Marcos et al., 2016). Under these pH conditions the carboxylase group of the alginate becomes protonated and form hydrogen bonds.

Even though, increasing the molecular weight of the alginate improves the physical properties of the alginate it is often undesirable in the processing as the polymer becomes highly viscous 18. However, when combined the high and low molecular weight alginate polymers the elasticity can be improved by slightly rising the viscosity of the solution (Kong et al., 2002).

The alginate that is available commercially is generally extracted from the brown algae phaeophyceae) like *Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum nodosum, and Macrocystis pyrifera (Smidsrød and Skja, 1990),* treating with aqueous alkali solutions usually NaOH (Clark and Green, 1936). The extract then is filtered and treated with either sodium or calcium chloride to precipitate it as alginate. This is treated with HCl to make alginic acid. After further purifications water soluble alginate powder is obtained. Different biomass of different seaweeds give different proportion of alginate (*A. nosodum* 22- 30% and L. *digitata* 25-44 %) (Qin, 2008).

1.3. Carrageenan

Carrageenan are the family of seaweed polysaccharides obtained from various species of red seaweeds known as rhodophyceae. They are sulfated and water soluble galactans are composed of alternate 3-linked beta-D-galactopyranose and 4-linked 3,6 anhydro-galactose and classified according to the number of position of sulfate group and 3,6 anhydro bridges in alpha-linked residues (Kalitnik et al., 2013). These naturally available polymers have ability to form thermo-reversible gels of viscous solutions when treated with salt solutions (Lahaye, 2001). The gel

obtained can be heavily used in texturing, thickening, suspending, and stabilizing agents in various industrial uses in food and pharmaceuticals (Hilliou et al., 2006).

Kappa-carrageenan and iota-carrageenan are carrageenan they can form gel. Iota-carrageenan forms a helicoidal secondary structure which is essential component of gel formation (Campo et al., 2009). Studies have suggested that iota-carrageenan with additional sulfate group in anhydrous galactose, exhibit greater hydrophilic properties than those of kappa carrageenan (Silva et al., 2010).

Carrageenan are mainly categorized into three types namely k-carrageenan, i-carrageenan and λ-carrageenan. This carrageenan have identical chemical structures and they all possess gelling and viscous improving properties. For example, k-carrageenan is less sulfated polymer and has strong and elastic gel making ability and shows thermal hysteresis. In contrary, λ-carrageenan has more sulfated structure and lacks helix forming property in solution and does not show gelling property (Hilliou et al., 2006). The carrageenan biopolymers are made up of combination of k-monomers and i-monomer (hybrid polymers) (van de Velde et al., 2001).

K-carrageenan contains 25-30% esters sulfate and 28-35% annhydrogalactose. Similarly, carrageenan contains 28-30% of ester sulfate and 25-30% annhydrogalactose unit. However, λ-carrageenan has more sulfate composition about 32-39% and no anhydrogalactose (Michel et al., 2001). Among the above mentioned types only k-carrageenan have firm, elastic and soft gel forming capacity on compared to that obtained from i- carrageenan (dos Santos and Grenha, 2015).

Fig 1.4. Structure of different types of carrageenan (Yegappan et al., 2018).

Carrageenan that are available commercially have the average molecular weight ranging from 100 to 1000 kDa. The pKa value, that determines the degree of ionization in different media, is about 2.0. K-carrageenan undergo the thermally-induced disordered-ordered transition, where at raised temperatures it exists as random coil (Li et al., 2014).

Carrageenan have many potential applications. They can be used in pharmaceutical and food industries as anticoagulant, anticancer and functional foods and preservatives. (Campo et al., 2009). Carrageenan have shown the antioxidant and free radical scavenging properties and it was found that there is positive correlation between sulfate content of the carrageenan and antioxidant activity(de Souza et al., 2007).

1.4. Making hydrogels

Chemical and physical cross linking of the hydrophilic polymers are the key approaches to make hydrogels. There are other various ways that hydrogels are made for application in various areas like food and biomedicals.

1.4.1. Ionic Cross-linking: This is the most commonly used procedure to make hydrogels from the alginate solution. Here, the alginate solution is mixed with the ionic cross-linking agents like divalent cations (Ca^{++}). These cations bind solely to guluronate blocks of alginate chain as these blocks permits high degree of coordination of divalent ions. The most common agent used in the cross linking of alginate is calcium chloride, but it basically leads to poorly controlled gelation due to its high solubility. This can be overcome by utilizing the buffer containing phosphate. The phosphate group in contained in the buffer solution competes with the carboxylate groups of alginate in the reaction with calcium ions and retard gelation (Crow and Nelson, 2006).

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Fig 1.5: Alginate hydrogels made by ionic cross-linking with calcium ions. (egg-box model) (Lee and Yuk, 2007)

1.4.2. Covalent Cross-linking: This is another approach that has been extensively studied to make improvements in the physical properties of the gel for many applications. However, covalent cross-linking arrangements may be toxic, so the unreacted chemicals should be thoroughly removed from the gels.

1.4.3. Thermal gelation: In many drug deliveries, heat-sensitive (thermo-sensitive) hydrogels have been extensively studied. The reason behind this is their changeable swelling properties in response to different temperatures (Roy et al., 2010). The more widely used gels are poly (N-isopropylacrylamide) hydrogels and these hydrogels undergo phase transition near the body temperature, at about 32^{o}C. The temperature may be changed by copolymerization with other hydrophilic components like acrylic acids and acrylamides (Rzaev et al., 2007). However, very few systems using alginate have been reported as they are less thermosensitive. But, semi-interpenetrating polymer network (semi-IPN) structures were made via polymerization on N-isopropylacrylamide with polyethylene glycol in presence of UV radiation. The swelling property of the gels increased as the concentration of alginate at certain temperature and decreased when the temperature increased (Zhao et al., 2010).

1.5. Biodegradation of alginate and its derivatives

As the mammals do not have enzyme called 'alginase', it is non-degradable in mammals. Alginase is an enzyme that can cut the polymer chain. However, alginate can be degraded ionically by releasing sodium ions around the surrounding areas. Although the gel is dissolved and degraded, it has been found that the molecular weight of many commercially available alginates are more than the renal clearance threshold of the kidneys, and there is chance that alginates cannot be completely removed from the body (Al-Shamkhani and Duncan, 1995). However, there is a very applicable approach to degrade the alginate in the body that is oxidation of alginate chains. Slightly oxidized alginates can degrade into the aqueous conditions, and this can be done by using sodium periodate. This oxidation cuts

the C-C bond of the cis-diol group in the uronate residue and changes the conformation to an open chain that makes the alginate degradable. The result is found to be effective if the molecular weight of the alginate is lowered during the oxidation. Interestingly, this slight oxidation of alginate does not affect the gel forming property of the alginate (Bouhadir et al., 2001).

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a. Partially oxidized alginate b. alginate degradation over time

Fig 1.6: Partially oxidized alginate and its degradation behavior. (Copyright 2001, John Wiley & Sons, New York, USA.*)*

1.6. Need and development of blended polymers

For developing a novel and biodegradable material having suitable chemical and physical properties for application in food and medical industries are now of immense interest. This could be achieved by blending alginates and carrageenan with other naturally occurring polymers like chitosan, starch, cellulose chitin or alginate-carrageenan mix (Zia et al., 2017). However, fewer literatures are available on the gel obtained by mixing alginate and carrageenan. So, this could area of interest to obtain a noble gelling material. In a study conducted by Pérez-Mateos et al. found that the mixing of k-carrageenan with sodium alginate showed thermally strong synergistic interaction without altering other functional properties of the gel. The basic mechanism behind the new blended polymer formation is that when these

polysaccharides are mixed at certain proportion, they form inter-penetrating polymer network with mechanically and thermally stable hybrid polysaccharide network (Kulkarni et al., 2011).

(Mao et al., 2012) depolymerized the alginate using oxidative degradation method. They used sodium alginate of molecular weight 346 kDa and depolymerized it using hydrogen peroxide at specified temperatures at different time frames of 0.5 h, 1 h, 2 h, 3 h and 4 h and alginate was recovered again by ethanol precipitation method using centrifuge and washing with deionized water and determined the molecular weight of the depolymerized alginate. They found that depolymerization of seaweed polysaccharide like alginate can give smaller molecular weight oligomers with increased stability. The research used 1% w/v alginate and 3% v/v hydrogen peroxide at system pH of 6. Reaction temperature was kept 20 °C and time were 0.5, 1, 3, and 4 hours. This leaves the possibility that the reaction time and temperature can be changed to find further results. This research is based on this finding of depolymerized alginate however they did not make any hybrid gels. This research will make hybrid gels using certain proportion of alginate and k-carrageenan and determine their molecular weight, thermal stability, elasticity and other physical and chemical properties before and after the depolymerization. This could produce a different results and findings than past experiments.

Table 1.5: Incorporation of various compounds in seaweed polysaccharides (Khalil et al., 2017) to enhance their properties.

Alginate	**Kappa-carrageenan**	**Agar**
Calcium chloride/glycerine	Grapefruit seed extract (GSE)/ glycerol	Arabinoxylan/glycerol
Apple puree and essential oil	Zataria multiflora essential oil and nanoclay/glycerol	Starch/glycerol

Sago starch and lemongrass oil/glycerol	Clay mineral and silver particles/glycerol	Silver nanoparticles (Ag)
Montmorillonite (MMT)	Chitin nanofibrils (CNF)	Nanoclay/glycerine
Cinnamon bark oil and soybean oil/glycerol	Silver nanoparticles (Ag) and PVP/PEG	Grapefruit seed extract (GSE)/ glycerol
Kappa- and Iota-carrageenan/glycerol		Banana powder and Silver (Ag) nanoparticles/glycerol
Silver nanoparticles/glycerol		Fish gelatine and TiO_2 nanoparticles

There have been several researches carried on improving the quality of the seaweed polysaccharides by incorporating with other compounds, however limited literature is available regarding the alginate-k-carrageenan mixed gels.

1.7. Mechanism of sodium alginate oxidation

Hydrogen peroxide oxidation is effective method in depolymerization of alginate rate of which is dependent on reaction time, hydrogen peroxide concentration, system pH and temperature. This give the depolymerized alginate of low molecular weight and low viscosity. There are several ways that can degrade alginate polysaccharides like introducing degradable polysaccharides into alginate or breaking the alginate backbone using acid hydrolysis by oxidation or enzymatic degradation (Li et al., 2010).

Hydrogen peroxide is an effective oxidizing agent which is environmentally safe and has been used to oxidize several polysaccharides like alginate and chitosan (Qin et al., 2002). This approach not only depolymerize the polysaccharide, it also change the chain structure

(Zeronian and Inglesby, 1995). In starch, hydrogen peroxide oxidation introduces the carboxyl and carbonyl groups and deamination. However, there are very few findings about the oxidation of alginate using hydrogen peroxide. When oxidized, the glucoside bonds in alginate break giving rise to low molecular and low viscosity alginate. It also forms the aldehyde group as a result of reduction reaction in C-1 (Li et al., 2010).

Fig.1.8 General mechanism of oxidation mechanism of alginate like polysaccharides a. Terminal (non-reducing end) residues: double oxidation between C2 and C3 and between C3 and C4, respectively, with the release of C3 as formaldehyde, (b) (1→4)-linked residues where cleavage occurs between C2 and C3, (c) (1→3)-linked residues (non-terminal), which are resistant to oxidation (Kristiansen et al., 2010).

1.8. Molecular weight and biocompatibility of the seaweed polysaccharides

The biocompatibility of alginate has been thoroughly studied under *in-vitro* as well *in-vivo,* however there is still debate considering the effect of alginate composition and this debate is linked to the level of purity of the alginate. For instance, it has been found that alginate with high M content are immunogenic and ten times more potential in compare to the high G containing alginates (Otterlei et al., 1991). As the alginates are natural sourced there is high chance they are contaminated with the various impurities like heavy metals, endotoxins, polyphenolic compounds and proteins. The alginate which is purified to high purity have no significant immunogenic reaction in the body (Lee and Lee, 2009). Seaweed polysaccharides like alginates are being extensively in various biomedical and food industries. As these polysaccharides degrade very slowly and uncontrollably and release high molecular weight strands that makes body hard to clear appropriately. So, there is high demand of lower and biocompatible molecular weight seaweed polysaccharides (Bouhadir et al., 2001). There are several approaches made to reduce the molecular weight of seaweed polysaccharides and oxidative degradation of these polysaccharides are proven to be more effective (Bouhadir et al., 2001) (Mao et al., 2012).

1.9. Seaweed polysaccharide market future perspectives

Majority of the global carrageenan (almost 90 %) are cultivated mainly in Indonesia and the Philippines. Other export market has also been obtained in Malaysia, Zanzibar and in the United Republic of Tanzania and Madagascar. The commercial production of seaweed polysaccharides is now growing in the other regions of south-east Asia (Vietnam, Cambodia, Myanmar, East Timor and Southern China) (Campbell and Hotchkiss, 2017). So, it looks there will be significant production of seaweed polysaccharides in the countries where ocean are their part countries. Australia has enormous potential regarding marine products development

in the future as the country is making millions of investments in coming decades as 'Blue Economy as the marine industries have grown rapidly. Also, after the years of consultation and priority development, the marine science community and government and stakeholders have developed and updated science plan to guide the next decade of progress in marine environment – 'National Marine Science Plan 2015-2025: driving the Australia's Blue Economy' (National Marine Science Committee 2015). Marine industries contributed $47 billion to the Australian economy during 2011-2012 and with rise in offshore gas production including marine biotechnology it is estimated to grow to $100 billion by 2025. This can be linked to the growing investment in marine industries including marine biotechnology (Treloar et al., 2016).

Seaweed polysaccharides are being widely used in industries for food and non-food applications. Seaweed produce alginate and carrageenan are used in food industries in gelling and thickening agent. And the polysaccharides are also used in encapsulation of drugs and functional foods. Sulfated polysaccharides from seaweed have application as drug for antiviral and antitumor activities (Cosenza et al., 2017).

1.10. Use of mixed seaweed polysaccharides in encapsulations

Functional foods such as fish oil have shown very good health benefits, and their benefits are more than the traditional approaches of food nutrition. Studies suggest that the regular or increased consumption of these functional foods may promote the general health and well-being and also reduce the risk of chronic diseases (Sun-Waterhouse, 2011). However, these bioactive compounds derived from marine microorganisms have problem in production and storage as they have high chance of worsening because of unsaturated fatty acid contained in them. Compounds like fish oil containing these fatty acids and other free radicals have high chance of degradation and reduces the self-life (Chang and Nickerson, 2018).

So, there is growing need of development of the techniques that can improve the stability and self-life span of bioactive compounds. For this approach the technique of encapsulation and microencapsulation have been in use recently (Comunian and Favaro-Trindade, 2016). In microencapsulation process, the sensitive bioactive compounds are entrapped into the wall material that can protect the material and also enhances the way of the delivery (Zhang et al., 2018). The encapsulation process saves the sensitive bioactive compounds from oxidation, heat and moisture. In case of fish oil, the encapsulation process can enhance the taste and can also reduce the smell of fish oil (Rivas et al., 2017).

Fig. 1.7 Representation of microencapsulation (Bakry et al., 2016).

Encapsulation using the mixture of two different polymers can have significant improvement in the stability of encapsulated beads and can reduce the leaking of incapsulated bioactive compound. This could be achieved by blending alginates and carrageenan with other naturally occurring polymers like chitosan, starch, cellulose chitin or alginate-carrageenan mix (Zia et al., 2017). However, fewer literatures are available on the gel obtained by mixing alginate and carrageenan. So, this could be he huge area of interest to obtain a noble gelling material. In a study conducted by (Pérez-Mateos et al., 2001) found that the mixing of k-carrageenan with

sodium alginate showed thermally strong synergistic interaction without altering other functional properties of the gel. Use of this alginate-carrageenan so can form a stable gel that an reduce the leakage of the bioactive.

1.11. Research gap and biotechnological significance of the research

Even though alginate and carrageenan are the naturally occurring polysaccharides its slow and uncontrollable degradation can sometime be unwanted feature (Boontheekul et al., 2005). Moreover, the development of desirable molecular weight polysaccharide that should be kept in mind for effective encapsulation of the products. This research will work on improving the various properties and problems of the seaweed polysaccharides and work to develop a technique to lower the molecular weight of the extracted polysaccharide to make a dominant commercial seaweed product in the prevailing market.

This research project is set up based on the researches done on the past by previous researchers. However, there are few researches and literatures on the hybrid polysaccharides made by using alginate and carrageenan. Moreover, are area of marine biotechnology being unfathomable specially in context of its possibility and diversity.

1.12. Hypothesis/Research question

"Oxidizing the high molecular weight and high viscosity alginate can reduce the molecular weight and viscosity of that alginate and this modified alginate incorporation with carrageenan can significantly reduce the oil leaking problem during encapsulation of fish oil".

1.13. Objectives

- *AIM 1: To reduce the molecular weight of marine based polysaccharide (sodium alginate) using H_2O_2 oxidative method*
- *AIM 2: To optimise the Oxidation conditions of alginate*
  - *Effect of time*
  - *H_2O_2 concentration*

AIM 3: To develop cross-linked polymer hybrids (alginate and carrageenan) suitable for industrial application (fish oil encapsulation)

CHAPTER 2: MATERIAL AND METHODS

Materials and Methods

2.1. Oxidation of sodium alginate using hydrogen peroxide to lower its molecular weight and viscosity

Sodium alginate medium viscosity (593 mPas and MW 824 kDa), and hydrogen peroxide 30% (v/v) obtained from Sigma Aldrich. Oxidation of the sodium alginate was done in controlled time and concentration of the hydrogen peroxide. Different concentration of the hydrogen peroxide (0.3% v/v, 0.5% v/v, and 1% v/v) were added slowly and dropwise in 1% (v/v) solution of sodium-alginate under the fume hood with continuous magmatic stirring (350 rpm). The reactions were allowed to take place for various time period; 30 min, 1 h, 2 h and 3 h. The oxidation reactions were stopped precipitating with double volume of 90% (v/v) ethanol. Then the precipitates were filtered using sieve and washed with distilled water to remove remaining hydrogen peroxide and ethanol. The precipitates were kept in -80^{o}C for 24 h and finally freeze dried for another 24 h (Bouhadir et al., 2001) and (Mao et al., 2012). Finally, oxidized (modified) alginate in powdery form was recovered and again 1% (w/v) solution was prepared from the modified alginate and its molecular weight and the viscosity were measured using HPLC and viscometer NDJ-8S Digital Rotary Viscometer (Rinch Industrial Co. Ltd., China). Solubility and MG ratio of the modified alginate were determined. Refer to 2.7 and 2.8 of methodology section for detailed explanation of procedure.

2.2. Preparation of alginate-carrageenan mixed polysaccharides films

Alginate-carrageenan mixed gels were prepared to study the various physical properties (gel strength, swelling ratio) of the resulting gels based on the finding that when two polysaccharides are mixed it forms a polymer network that is more stable and stronger than the single polysaccharide gel. The following proportions of alginate (Sigma Aldrich) and

carrageenan (obtained from GGOG China) were mixed to make the final concentration of 1% (w/v) stirring vigorously using magnetic stirrer.

Table 2.1: Mixing of different amount of alginate and carrageenan

Sod-alginate	100 mg	200 mg	500 mg	400 mg	300 mg
carrageenan	900 mg	800 mg	500 mg	600 mg	700 mg

The above-mentioned amount was weighed and mixed together in distilled water (100 mL) mixing vigorously using them magnetic stirrer at 350 rpm. After complete mixing 50 mL of the alginate-carrageenan solution were poured into a perti plate and allowed to dry in oven for 24 hours in temperature of 50 °C. After 24 h the alginate-carrageenan mixed films were taken out and soaked in 0.5% (w/v) calcium chloride solution with 20% (v/v) ethanol for 30 min. Ethanol was used to enhance the crosslinking of the gels (Mao et al., 2012). Then the films were dried and used for studying its properties. The physical properties of the hybrid gel was studied.

2.3. Preparation of fish oil encapsulated beads using alginate-carrageenan mixed gels

This methodology is based on the methodology described by Chan (Chan 2011). To calculate the encapsulation efficiency, 250 mL conical flask was taken and was labelled as W_1. Total volume of 1% (w/v) mixture (0.3% w/v alginate + 0.7% w/v carrageenan) was made by adding 90 mL of distilled water and 10 mL (9.3g =10 mL fish oil) of fish oil and total weight was taken and labelled as W_i. All the weight with containing the glass bottle was labelled as W_2. Then the final mixture was emulsified using ultrasonic mixture under room temperature. The emulsion was transferred to a syringe with a needle and weight was taken as W_a. Emulsion

solution was dropped into 100 mL of 150mM calcium chloride solution while continuous stirring with magnetic stirrer at 150 rpm at room temperature 23 ^{o}C. The empty syringe and needle (23 x G) were weighed again after complete dropping of emulsion solution and labelled as W_b. The beads formed in the $CaCl_2$ solution were incubated for 1 h to allow good crosslinking with the calcium. After 1h incubation the beads were filtered with the help of sieve.

***Calculation* of encapsulation efficiency:**

Weight of emulsion solution $W_e = W_2 - W_1$

Amount of oil in the emulsion $C_0 = W_i / W_e$

Actual emulsion weight into the gelling solution $W_d = W_a - W_b$

Fish oil into the gelling solution= C0-Wd, this equation was used to calculate the efficiency of encapsulation. This methodology is based on the methodology developed by Chan 2011, Kim et al., 2008.

2.4. Determination of encapsulation efficiency

After encapsulation the beads were separated using sieve from the $CaCl_2$ solution, and hexane 20 mL was added to the $CaCl_2$ solution, the remaining oil in the solution was separated and absorbance was determined at 269 nm, as OD_1. The oil content was calculated by using the standard curve. Similarly, the beads were also washed with 20 mL hexane to extract the surface oil as the oil is soluble in hexane, and again absorbance was taken at same wavelength and marked as OD_2. Again, standard curve (see appendix) was used to calculate the amount of surface oil. This method is based on the procedure described by (Bannikova et al., 2018) and Chattergee and Judeh (Chattergee and Judeh, 2015)

Calculation:

The encapsulation efficiency EE= Initial oil content - (oil in the gelling solution - oil in the surface of the beads)/Initial oil content] x 100%

2.5. Stability of encapsulated beads

To determine the beads stability freeze, dried beads were stored in Petri dish packed by parafilm to isolate the beads away from air. The weight of Petri dishes was measure before use. These Petri dishes contained beads were incubated for 7 days in desiccator at room temperature. The oil leaking was measured by determining the surface oil and the oil on Petri dish surface. Beads were transferred into a glass vial, shaking with 20 mL hexane (hexane can extract oil from washing solution) for 1 min. The amount of oil leaking on beads surface was determined by UV-spectroscopy, the same as the encapsulation efficiency test. By the standard curve, the surface oil was calculated as WS, the oil on surface of container was weighted as WC by determination the change of the Petri dish.

$WL = WS + WC$

$Oil\ leaking\ (\%) = [WL / WO] \times 100\%$

WL was the total amount of oil leaking in 7 days.

The formula is modified form the methods of Bannikova et al (Bannikova et al., 2018) and Chatterjee and Judeh (Chatterjee and Judeh, 2015) and modified by Zhaolin He, Masters student at Flinders University.

2.6. Equilibrium swelling ratio of the alginate-carrageenan mixed biofilms

Among the different biofilms prepared, the most flexible, and transparent one was selected to study the swelling equilibrium ratio. The dry film was weight first and was soaked in water (50 mL). The weight of the wet gels was measured at different time to obtain the swelling

equilibrium. Before weighing the wet films, the excess surface water was soaked with filter paper and welling ratio of the 1% (w/v) alginate-carrageenan film was compared.

The equilibrium swelling ratio (S_{eq}) of the alginate-carrageenan mixed biofilm was calculated by the following equation:

$$S_{eq} = [(W_e - W_D)/W_D] \times 100\%,$$

Where, W_e is the weight of the swollen film, and W_D is the weight of the corresponding dry film at time $t=0$

All the swelling ratio of different biofilms were at different time were done in 2 replicates, n=2.

The swelling ratio (S_r) of the alginate-carrageenan mixed films, at time *t*, was calculated using following equation;

$$S_r = [(W_t - W_D)/W_D] \times 100\%,$$

This methodology is based on the process describe by (Kim et al., 1992).

2.7. Mechanical properties of alginate-carrageenan mixed biofilms

The mechanical properties of the prepared alginate-carrageenan biofilm were studied using DMA Q800 V7.4 Build 126 technique (Japan). Approximately 1×1 cm of the prepared dry biofilm (rectangular) piece was sent to study its mechanical properties like loss modulus, stress and stiffness under the influence of time and temperature.

2.8. Determination of molecular weight of oxidized alginates

The molecular weight distributions of oxidized sodium alginate at different oxidation time and hydrogen peroxide concentration were analysed using size-exclusion chromatography using Prominence UHPLC (Shimadzu- LC20A XR system, Japan) equipped with refractive index detector (RID-10A, Shimadzu, Japan). The size separations were performed using in-line

PolySep GFCP5000 and PolySep-GFC-P6000 columns (Phenomenex, USA). The oxidized alginate solution was mixed with 0.1M sodium nitrate at a concentration of 5 mg/mL. The samples were allowed to dissolve overnight in room temperature at 150 rpm using a bench top orbital shaker (model-O0M15, Ratek, VIC, Australia) to get a uniform mixture and later centrifuged (11,336Å~g). The obtained supernatant was thereafter injected into the HPLC system. The analysis was conducted at room temperature with 0.1M sodium nitrate as the mobile phase at a flow rate 1 mL/min. All the chemicals and standards used in this study were of analytical grade. The run was performed in duplicates and the results were presented as average values with standard deviations (±SD). The column was calibrated with standard dextrans obtained from Sigma (peak MWs of 65, 195, 400, and 1050 kDa).

2.10. Determination of M/G ratios, viscosity and solubility of oxidized alginate

The mannuronic acid (M) and guluronic acid (G) ratio (M/G) of the oxidized were determined by estimating the guluronic acid and mannuronic acid in the polysaccharides. The samples were prepared by following the method from (Abraham et al., 2019) using a Prominence UHPLC (Shimadzu- LC20A XR system, Japan) instrument with a Prominence SPD-20A UV–vis Detector (Shimadzu, Japan). The freeze-dried oxidized alginate (10 mg) were dissolved in 72% (v/v) sulphuric acid and incubated for 1 h at room temperature and later treated with 1M sulphuric acid at 100 °C for 3 h. The released monosaccharides were then subjected to derivatization with 1-phenyl-3-methyl-5-pyrazolone and the derivatization protocol was followed exactly according to (Abraham et al., 2019) and (Comino et al., 2013). The analysis was performed using Kinetex 2.6u C18 100A, 100Å~3 mm, Phenomenex column (USA), guluronic acid and mannuronic acid standards from Sigma-Aldrich. The other biochemical

properties of the extracted alginate were investigated by determining their viscosity and pH solubility range.

The viscosity of the polysaccharides was determined using a viscometer (NDJ-8S Digital Rotary Viscometer Rinch Industrial Co. Ltd., China) at 22-23 °C.

The solubility range of the H_2O_2 oxidized sodium alginate was determined by its stability as a solution from acid to alkaline pH range. The pH of the solution was determined by using pH meter (TPS 901-pH, Stennick Scientific, South Australia). The lowest pH was recorded when solution begins to precipitate. The experiment was conducted using 1% (w/v) oxidized alginate and done in duplicates (n=2)

2.11. Frequency size distribution of encapsulated beads

The diameters of 50 encapsulated dry beads were measured using scale and diameters were determined by using software ImageJ (Abràmoff et al., 2004) and graphs were prepared using GraphPad (Anderson et al., 2016) where diameter of fifty randomly taken beads were determined.

Experiments and tests were done at least two replicates (some even three replicates) and values were shown with mean and standard error. Standard curve of fish oil in hexane had shown linear relationship (R2 = 0.9998). Diameters of 50 randomly taken beads were measured to determine the size and shape of beads per sample.

CHAPTER 3: RESULTS AND DISCUSSIONS

3.1. Oxidation time vs viscosity

1% (w/v) alginate was oxidized by using different concentrations of hydrogen peroxide and the oxidation of the sodium alginate was initially studied my measuring the viscosity of these oxidized alginate in different conditions by viscometry. As shown in Fig 3.1, when 0.3% (v/v) hydrogen peroxide was used for oxidation for 30 min, 1 h, 2 h and 3 h, the viscosity of the solution was decreased as the reaction time was increased. The viscosity of the original sodium alginate (control) was 593 mPas, and this during the first 30 min of oxidation was decreased by almost 3 times (184 mPas). When the oxidation time was further increased the viscosity kept on decreasing. After 3 h of oxidation the viscosity was 130.2 mPas.

0.3% (v/v) hydrogen peroxide was used to oxidize 1% (w/v) Sigma sodium-alginate) at room temperature of 22-23^{o}C.

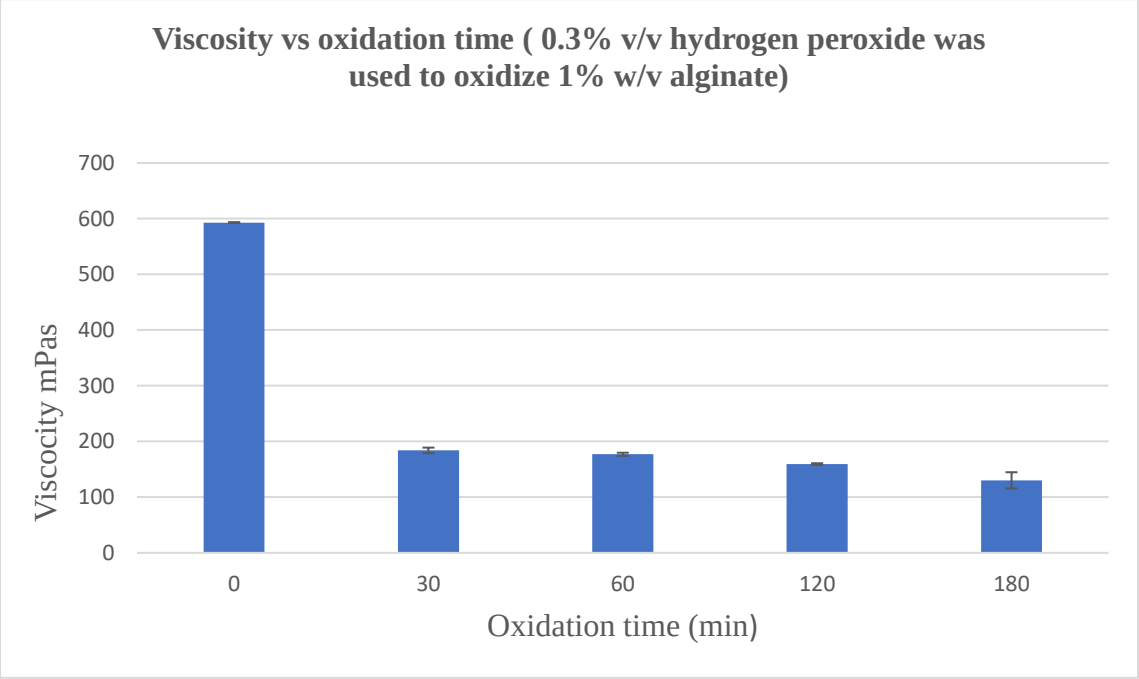

Fig 3.1: Graph showing viscosity vs oxidation time (0.3% (v/v) hydrogen peroxide* was used to oxidize 1% (v/v) Sod-alginate). As the oxidation time was increased, there was significant

decrease in the viscosity of the sodium alginate with original viscosity 593 mPas. n=3. *3.33 mL 30% hydrogen peroxide added in 96.7 mL of 1% (w/v) alginate solution was added for oxidation to make final volume of 1% (v/v).

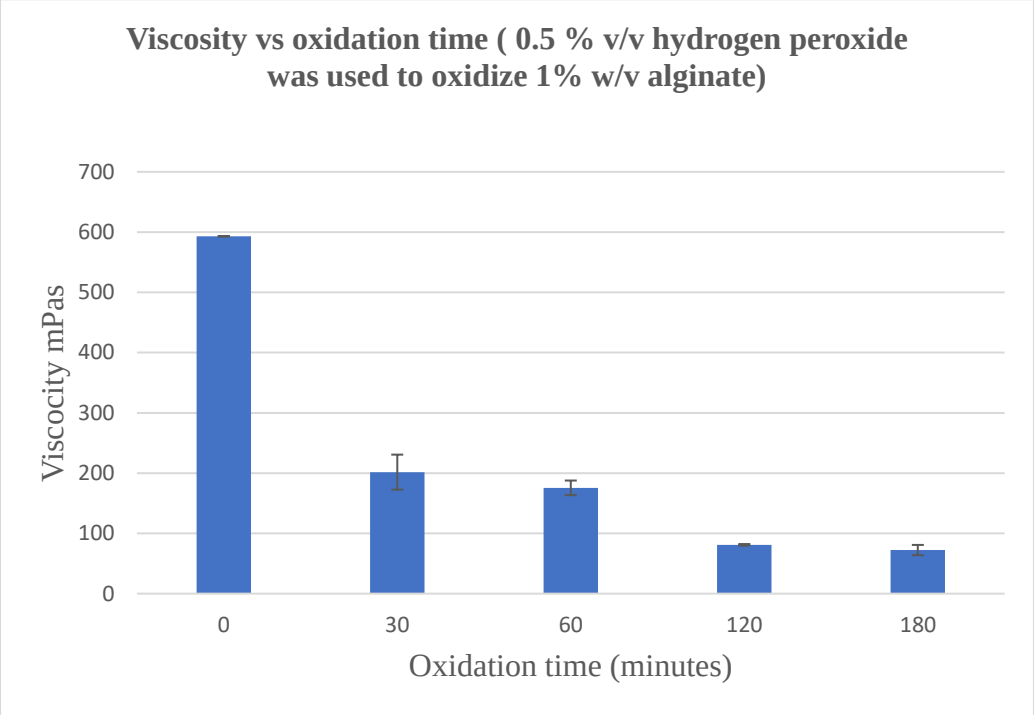

Fig 3.2: Graph showing viscosity vs oxidation time (0.5% hydrogen peroxide was used to oxidize 1% (w/v) Sod-alginate). As the oxidation time was increased, there was significant decrease in the viscosity of the sodium alginate with original viscosity 593 mPas.

During the second phase of the oxidation reaction, the concentration of oxidizing hydrogen peroxide was increased to 0.5% (v/v) and 1% (v/v) fig 3.2 and 3.3. The reaction time were kept same that is 30 min, 1 h, 2 h and 3 h. This increase in hydrogen peroxide concentration further decreased the viscosity of the sodium alginate. The decrease in the viscosity during the first 30 min of oxidation was comparable to the viscosity obtained by using 0.3% (v/v) hydrogen peroxide during the first set of the reaction but with the increase in the oxidation time the viscosity was significantly lower than the using 0.3% (v/v) hydrogen peroxide for oxidation. After the oxidation of 2 h and 3 h using 0.5% (v/v) hydrogen peroxide the viscosity was lowered

to 81.0 and 72.3 mPas respectively. Similarly, the viscosity of the sodium alginate solution was markedly decreased when the concentration of hydrogen peroxide was increased to 1% (v/v). The viscosity after first 30 min of the oxidation was 142.5 mPas. With the increased reaction time the viscosity was lowered to 41.2 mPas, which is actually a very low viscosity in compared to the original sodium alginate from Sigma Aldrich. Oxidation of alginate using hydrogen peroxide is found to be due to the free radicals probably formed from the decomposition of H_2O_2. The resulting free radicals are strong oxidants that can break the hydrogen atoms from the glycosidic bond of the alginate reforming the alginate molecule and breaking the glycosidic bonds lowering the viscosity of the resulting solution (Li, 2010). The hydroxyl free radicals abstract the C-1 hydrogen atom from the mannuronic acid at first and the molecule rearranges itself and repairs the broken glycosidic bond (Yang, 2004). However, the exact mechanism how the oxidation of alginate happens using H_2O_2 is not so clear.

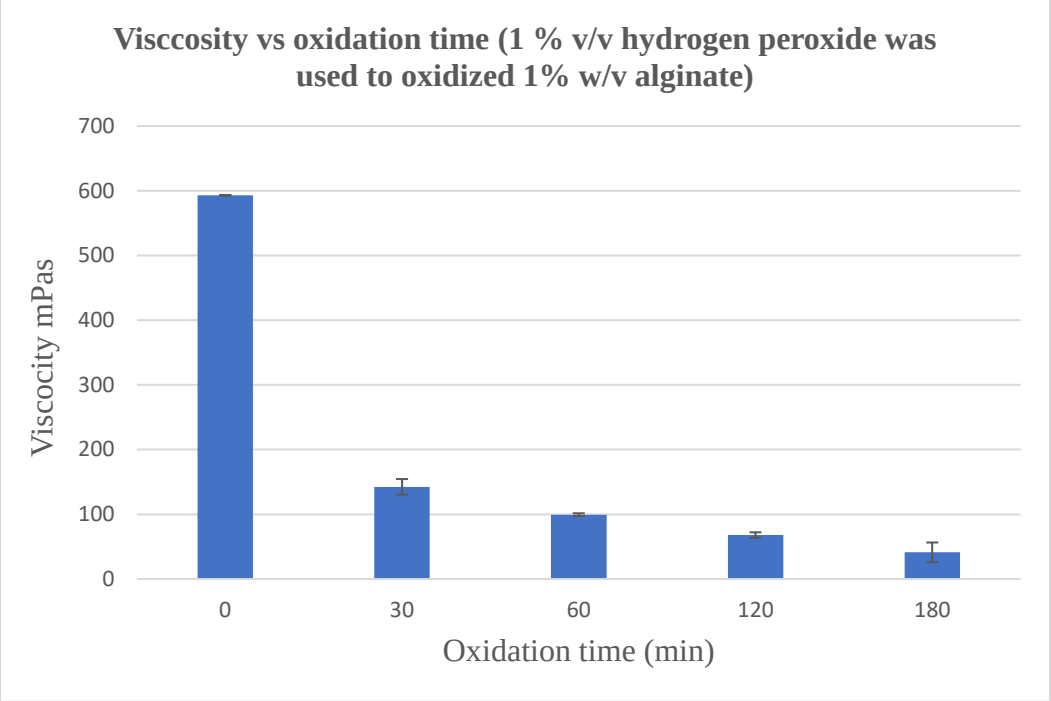

Fig 3.3: Oxidation vs viscosity (1 % v/v hydrogen peroxide was used to oxidize 1% w/v Sod-alginate). As the oxidation time was increased, there was significant decrease in the viscosity of the sodium alginate with original viscosity 593 mPas.

3.2. Oxidation time vs molecular weight.

Similarly, the molecular weight of the oxidized alginate was also studied. 0.5% (v/v) and 1% (v/v) H_2O_2 was used to oxidize the sodium alginate and their molecular weight at different oxidation time was determined. It was found that with the increase in H_2O_2 concentration and oxidation the molecular weight of sodium alginate was decreased slowly Fig 3.4 and 3.5. The molecular weight of the original alginate was 824 kDa and this was lowered to about 543.0 kDda during the first 30 min of oxidation. As the time was increased further decrease in molecular weight was observed. The molecular weight of 143.5 kDa was obtained after 1 hour of oxidation. Oxidation using 1% (v/v) H_2O_2 was more effective in reducing the molecular weight of the alginate. At this condition the molecular weight of the oxidized alginate was

recorded 478.4 kDa, 214.7 kDa, 190.3 kDa and 140.8 kDa during the oxidation time of 30 min, 1h, 2 h and 3 h respectively fig 3.5.

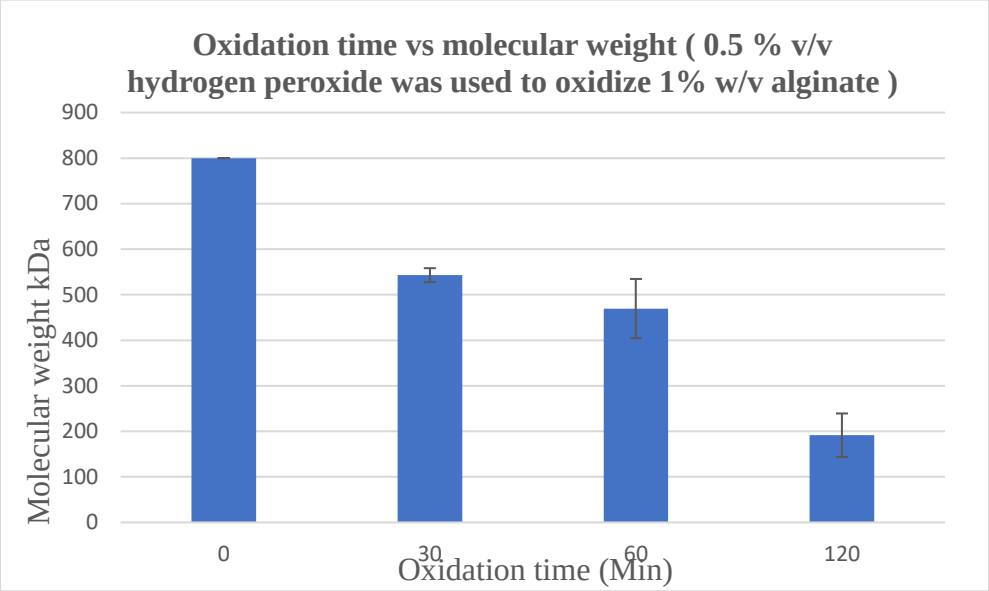

Fig 3.4: Time vs Mw (for 1% v/v hydrogen peroxide oxidation and 1% w/v alginate)

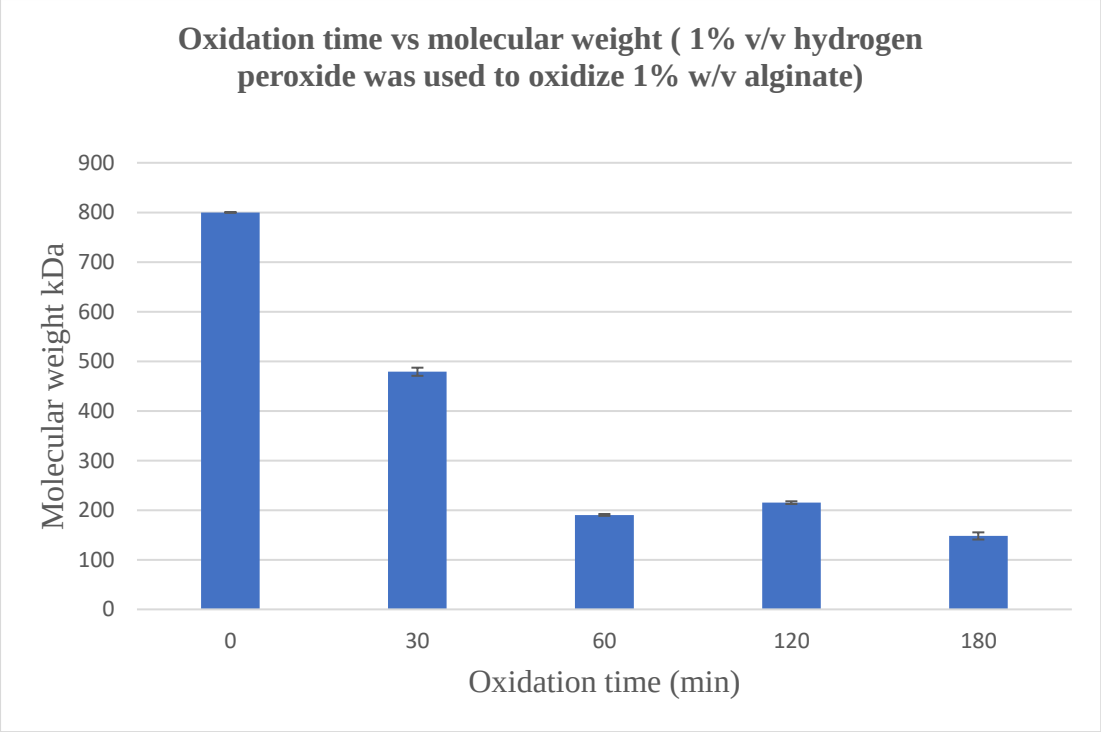

Fig 3.5: Oxidation time vs Molecular weight (1% v/v hydrogen peroxide for 1% w/v alginate).

0 represents the control alginate that is no hydrogen peroxide was used/ unoxidized

3.3. G/M ratio and solubility of oxidized alginate

G/M ratio of the oxidized alginate was determined using high performance liquid chromatography (HPLC). As shown in the table 3.1 the experiments were determined in triplicates. There was no significant difference between the G/M ratios between the original sodium alginate and the oxidized alginate. Also, the content of the ManAc and GluAc was similar. The G/M ratio of the original alginate was 0.320 and of the modified was 0.393. As the G/M ratio is important factor for alginate forming the gel this ratio shows that the oxidized alginate can also form gel like original alginate can form.

Solubility of oxidized alginate

When 1% (w/v) solution of modified alginate was casted dropwise in 1M HCl (hydrochloric acid) solution, the alginate started to precipitate at pH 5.0 and the complete precipitation was at pH 3.0- pH 2.97. The pH of the 1% (w/v) modified alginate was 6.5. This suggests that the modified (Oxidized) alginate is soluble in the mild pH and it completely solidifies at the lower pH which can be linked to biodegradability in human intestinal pH.

Table 3.1: G/M ratios of sodium alginate (control /unoxidized) and oxidised alginate and solubility of modified alginate. The G/M ratios of the unoxidized sodium alginate was referred from previous results of Peng Su, and Reinu Elsa Abraham. There was no significant difference between the G/M ratios of oxidized alginate and unoxidized alginate. It suggests that even the alginate was oxidized it has not lost its gelling property, it is like unoxidized alginate. G/M ratio plays important role in gelling properties of alginate. The experiments were done in triplicates n=3.

a. Solubility

pH of 1% (w/v) Oxidized Alginate	pH at which alginate starts to precipitate	pH at which the alginate becomes complete gel
6.5	5.0	3.0 - 2.97

b. G/M Ratios: G/M ratios of oxidized and unoxidized alginate (control), n=3

Sample	*GluAc*	*ManAc*	*G/M Ratio*
Unoxidized Sigma Sod-alginate *	14.45±1.34	45.09±3.04	**0.320**
Oxidized alginate	19.42±6.73	49.37±14.14	**0.393**

3.4 Alginate-carrageenan mixed films

Fig 3.6: Left: Hybrid gel formed by mixing 300 mg of sodium alginate (Sigma medium viscosity) and carrageenan 700 mg. The final concentration was 1% (w/v). The gel was strong

enough to hold in hand with excellent transparency. When mixed the polysaccharides form IPN inter-penetrating polymer networks which improves the strength and stability of the gel that can be used in encapsulation and food packaging. Right: Wet fish oil encapsulated beads using 0.3% modified alginate and 0.7% (w/v) carrageenan mixture 1% (w/v).

3.5. Viscosity of the resulting mixture

Table 3.2: Viscosity of different alginate carrageenan mixture (Total concentration 1% (w/v)). Viscosity of Sigma medium viscosity alginate; 593 mPas and GGOG's carrageenan 18080 mPas.

Alginate-carrageenan mixture (mg)	**Viscosity of final mixture mPas**
100mg+900mg	368.0
200mg+800mg	138.0
300mg+700mg	103.5
400mg+600mg	118.0
500mg+500mg	124.0
Viscosity of unoxidized alginate 1% (W/v)	593.0
Viscosity of carrageenan 1% (W/v)	18080

When alginate and carrageenan were mixed according to shown in table 3.2, the resulting viscosity of mixture showed low viscosity than the unoxidized alginate 1% (w/v) and carrageenan 1% (w/v). The viscosity of 1% (w/v) carrageenan was 18018 mPas but mixed with the alginate the mixture was less viscous. This is due to the fact that when a less viscous or

liquids of different viscosity are mixed the resulting viscosity is changed due to the effect of the some undergoing chemical reaction, even the final viscosity if influenced by the pH of the two different liquids (Nagatsu et al., 2007).

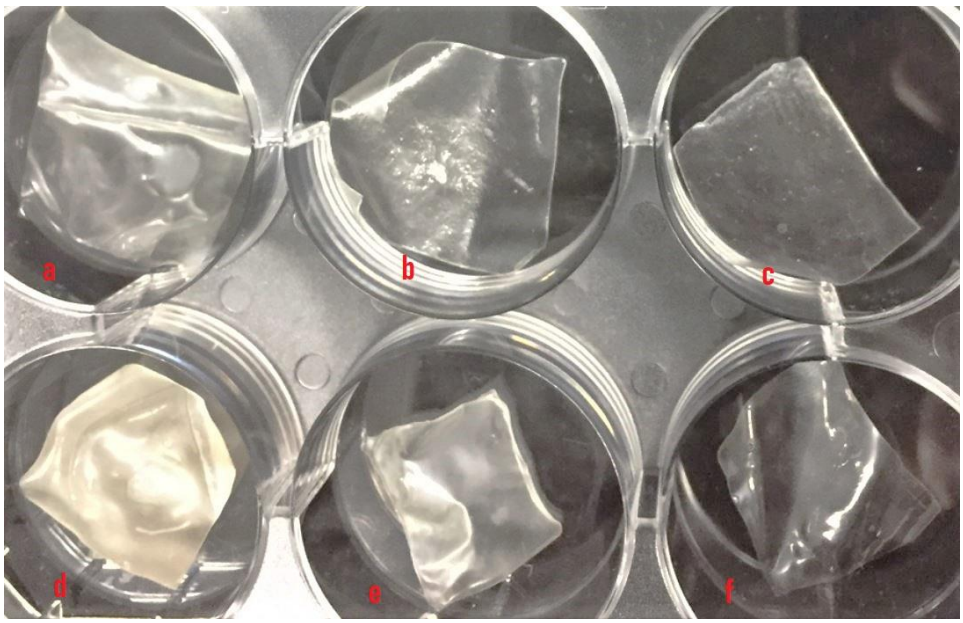

Fig 3.7: Alginate-carrageenan mixed polysaccharide biofilms. Various concentration of alginate and carrageenan gels were mixed to form different types of the biofilms. (a) Alg500+Carr300 mg mix, (b) Alg100+Carr900 mg mix, c only carrageenan (1% w/v), (d) Alg700+Carr300 mix, (e) Alg600+Carr400 mix, and (f) Alg300+Carr700.

Another aim of this experiment was to study the how the mixture of the alginate and carrageenan can form the gels and biofilms. We made different types of biofilms using certain proportion of alginate and carrageenan to study their physical properties for further application in commercial application fig 3.7. Total concentration of the mixture was 1% (v/v) mixing certain amount of Alginate and carrageenan. Among the biofilms made the on with 300 mg alginate plus 700 mg carrageen (fig 3.7, f) was selected for further study and application and its properties like strength and swelling ratios were studied. This hybrid biofilm showed good transparency and flexibility compared to other hybrid biofilms. They were not so transparent, cloudy and not flexible and were brittle.

3.6. Swelling Ratio of the biofilms

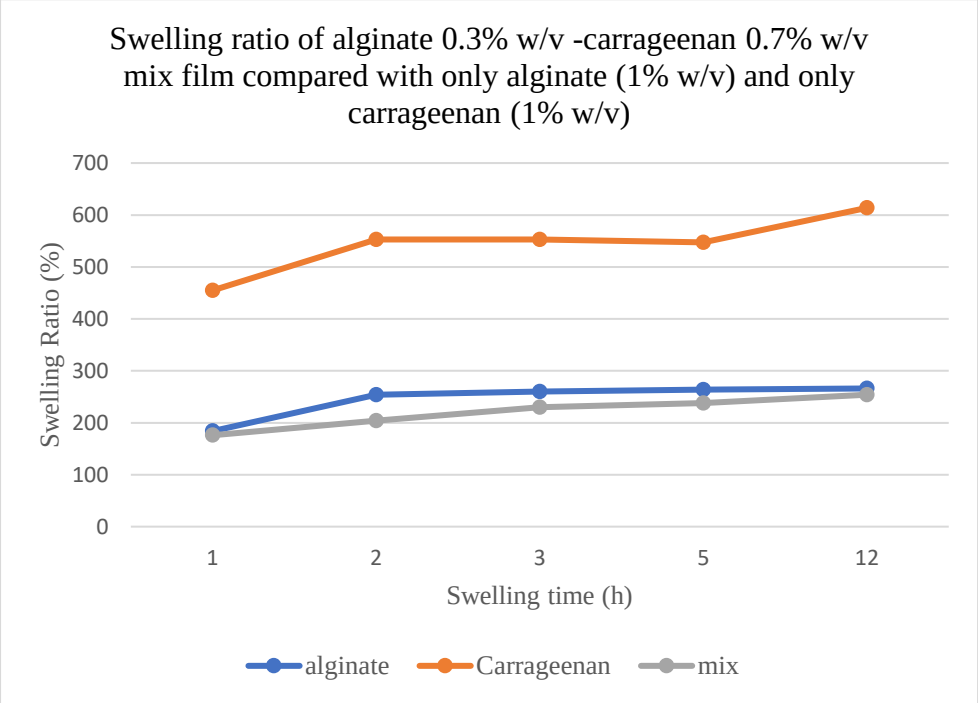

Fig 3.8: Swelling ratios of alginate-carrageenan (Alg300+Carr700) mg mixed film compared with 1% (w/v) alginate and 1% (w/v) carrageenan films

Swelling ratio of 1% (w/v) alginate, 1% (w/v) carrageenan and 1% (w/v) alginate-carrageenan (w/v) mix films were studied. The swelling in case of mixed polysaccharide biofilm was slow and steady, this is because, when the polysaccharides are mixed, they form a compact and strong network so that the water absorption becomes relatively slower than the single polysaccharide gels.

3.7. Physical properties of alginate-carrageenan mixed gels

Table 3.3: Loss modulus, stress and stiffness of the alginate-carrageenan biofilm on different time and temperatures.

MPa; Megapascal pressure unit, N/m: Newton per metre.

Temperature (ºC)	**Time (min)**	**Loss modulus (MPa)**	**Stress (MPa)**	**Stiffness (N/m)**
-100.3	0.98	235.18	4.15	469427.4
-80.95	8.28	218.3	4.1	463694.9
-60.89	14.93	211.74	3.85	435170.4
-40.88	21.63	219.75	3.54	400476.2
-20.89	28.28	230.74	3.18	359582.9
0.088	35.33	225.48	2.76	312547.8
20.1	41.98	216.9	2.37	268625.8
40.08	48.63	222.27	2.17	245249
60.21	55.33	219.51	2.21	250083.3
80.15	61.93	227.07	2.36	267049.2
100.14	68.03	255.94	2.5	282913.5

The biofilm showed very good thermostability. It can withstand the stress of 2.5 MPa even if the temperature was raised to 100 ºC. This can demonstrate that a very stable biofilm using alginate and carrageenan mixture at certain proportions can be prepared. As the stability of a gel is dependent on the final concentration of the mixture these properties can be further enhanced increasing the concentration of the mixture.

3.8. Encapsulation of fish oil using oxidized alginate incorporation with alginate and modified alginate

Mixture of alginate and carrageenan (1% v/v) and oxidized alginate 1% (w/v) oxidized with 1% (v/v) H_2O_2 for 1 h) was used to encapsulate fish oil (10% v/v) and the efficiency and stability was compared with 1% sodium alginate (control). As shown in the table 3.4 the encapsulation efficiency was around 98 % for all the alginate and alginate mixture. 1% w/v control alginate showed 98.71% efficiency and the modified encapsulated 97.06 % fish oil. 20% (v/v) ethanol was also used to see the effect of ethanol on the calcium-alginate network as some articles suggested ethanol enhances the crosslinking between the alginate and calcium ion present in gelling solution.

Table 3.4: Encapsulation efficiency of modified alginate mixed with carrageenan and medium viscosity alginate (Control) n=3

Beads (10% oil loading)	**EE (%) with STDEV**
0.3% medium viscosity alginate (unmodified) & 0.7% carrageenan	98.40±0.20
0.3% modified-alginate & 0.7% carrageenan	97.92 ±0.83
0.3% medium viscosity alginate + 0.7% carrageenan with 20% ethanol	97.60±0.28
0.3% modified-alginate + 0.7% carrageenan with 20% ethanol	97.94±0.12

As all the samples exhibited excellent encapsulation efficiency now the problem was to study if the beads formed are stable enough to hold the fish oil for certain period (1-week incubation was done in this experiment). So, test to determine the stability of the beads was done, Table 3.5. Oxidised alginate incorporation with carrageenan showed higher stability as the oil leaking at the end of week 1 was minimum than unoxidized alginate-carrageenan mixture. When 1% (w/v) sodium alginate (unmodified) and carrageenan mixture was used to encapsulate the fish oil, leaking was 29% of the total oil loaded. The most stable was 1% (w/v) mixture of oxidized alginate-carrageenan (oxidized-alginate 0.3% (w/v) + carrageenan 0.7% (w/v)). With this mixed alginate-carrageenan used encapsulation, the oil leakage was significantly reduced to about 13%. This stability is may be due to the polymer network formed between low viscosity, low molecular weight oxidized alginate and carrageenan. We also carried the encapsulation of modified alginate-carrageenan mixture in presence of 20% (v/v) ethanol as literature (Mao et al., 2012) suggest the ethanol enhances the crosslinking between polymer however the stability was lower the encapsulation without ethanol. The small oligosaccharide chain of modified alginate might have gone through the pores of big chain polysaccharide of carrageenan blocking the pores. So that, it formed stable network reducing the oil leakage. This network could be the IPN: Interpenetrating Polymer Networks that stabilizes the encapsulated beads. Further investigations are needed how these tow polymers form network within what is the structure of oxidized alginate looks like (Kulkarni et al., 2011).

Table 3.5: Stability of different fish oil encapsulated beads. Mixing oxidized alginate with other polysaccharide like carrageenan markedly improved the beads property and stability with minimal oil leaking of the oil after 7 days of incubation. n=3

Beads (10% oil loading)	**Oil leaking (%) with STDEV***
0.3% unoxidized alginate & 0.7% carrageenan	29.60±5.38
0.3% oxidized-alginate & 0.7% carrageenan mix	13.83±0.02
0.3% unoxidized-alginate + 0.7% carrageenan with 20% ethanol	21.80±9.99
0.3% oxidized-alginate + 0.7% carrageenan with 20% ethanol	18.22±5.60

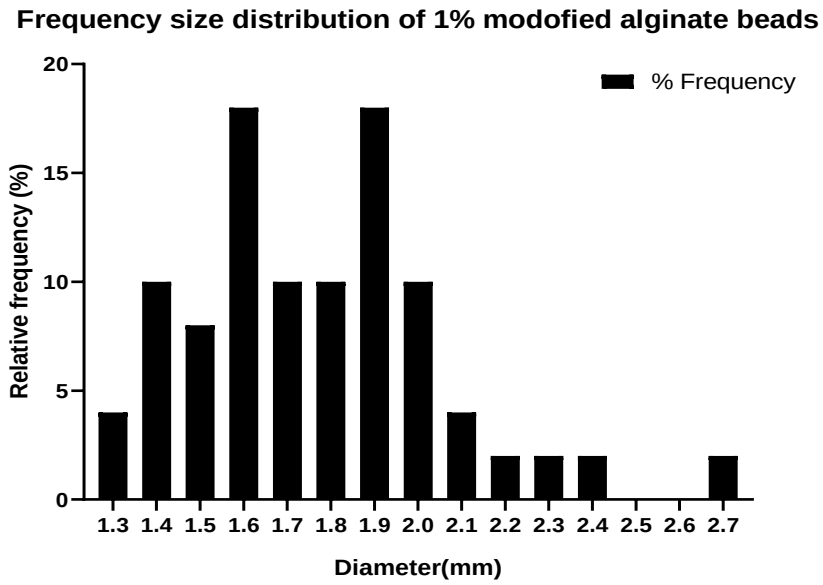

Fig 3.9: Frequency size distribution of oxidized alginate beads. 1% (v/v) hydrogen peroxide was used to oxidize 1% (v/v) medium viscosity alginate 1% (v/v) for 1 h)

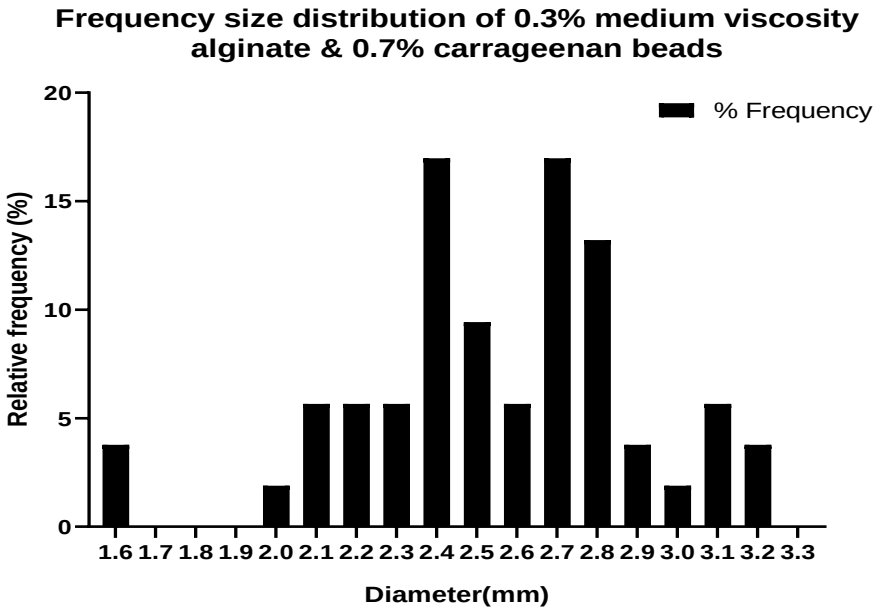

Fig 3.10: Frequency size distribution of alginate-carrageenan beads (0.3% (w/v) sodium alginate oxidized with 1% (v/v) hydrogen peroxide for 1 h was mixed with 0.7% (w/v) carrageenan to encapsulate the fish oil)

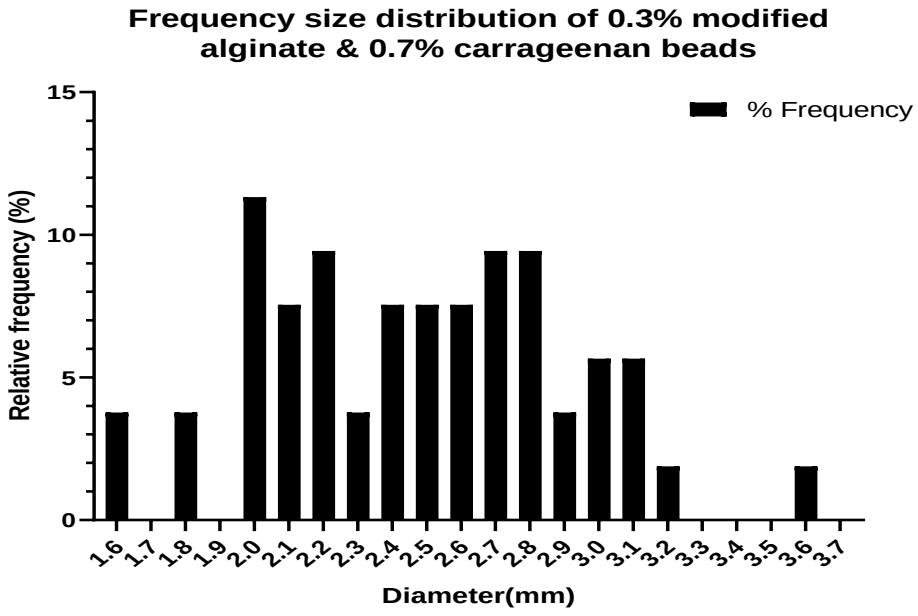

Fig 3.11: Frequency size distribution of oxidized alginate-carrageenan beads (0.3% (w/v) oxidized sodium alginate oxidized was mixed with 0.7% (w/v) carrageenan to encapsulate the fish oil)

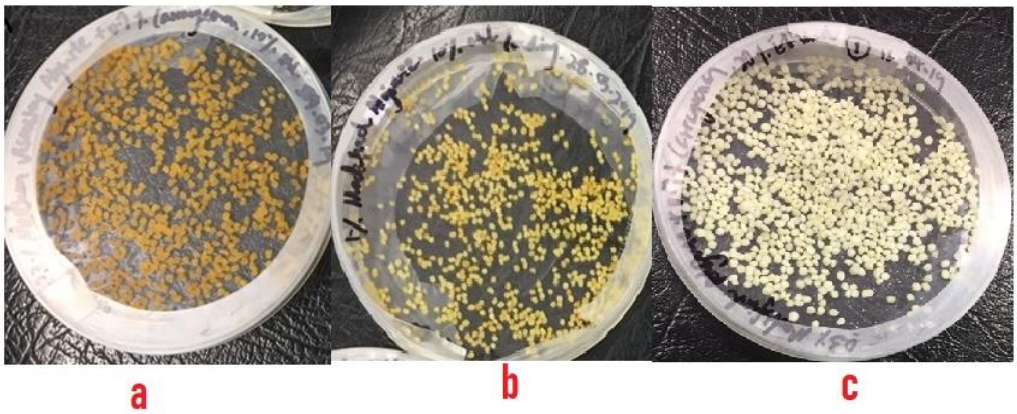

Fig 3.12: Fish oil encapsulated beads (10% v/v fish oil) after a week of incubation. (a) unoxidized alginate 0.3% (w/v) and carrageenan 0.7 (w/v) mix, (b) only oxidized alginate 1% (w/v), and (c) oxidized alginate 0.3% (w/v) + 0.7% (w/v) carrageenan mix. The oxidized-alginate and carrageenan mixture showed good stability, and other two turned yellow due to oil leaking.

3.9. Frequency size distribution of encapsulated beads

Size distribution of the encapsulated beads (Fig 3.7, 3.8, and 3.9) was studied. It was observed with carrageenan and only modified alginate were mostly between 2.0 mm to 3.0 mm. Whereas, the size of beads obtained from the sodium alginate (control) was between, 1.5 mm to 2.0 mm.

4. Conclusion

As the concentration of hydrogen peroxide in the oxidation of alginate increases it lowers the viscosity of the give alginate. Also, more the oxidation time lowers the viscosity. Not only the

viscosity molecular weight of the higher molecular weight alginates can be significantly lowered without making a lot difference in their G/M ratio and losing the alginates gelling capacity. Similarly, mixing alginate and carrageenan can form a very stable and strong biofilm that can have high commercial value and application in future. When we mix the lower viscosity and lower molecular weight oxidized alginate with another polysaccharide like carrageenan this could have very good application in drug encapsulation, in this case we use fish oil to encapsulate. In a nutshell, the physical and chemical properties of the existing high viscosity and high molecular weight alginates can be improved for commercial applications**.**

5. Incompleteness and limitations of the study

The main limitation of this study was that we were able to study only limited types of alginate. We worked on Sigma Aldrich Medium Viscosity alginate (Mw 824 kDa) and carrageenan. In further research other types of alginates and carrageenan be done to make the comparative study. Lack of enough time was another limitation of this study. During the nine-months short time period, coming with a solid idea that can be fully completed was a difficult task. Making proposals, reviewing the literatures and writing literature, and lab induction took another extra time. Some lab experiments were even week long and replicating and triplicating the results took even more time. We have developed an idea that seaweed polysaccharides can be used to make some biodegradable hybrid films and we even developed many different types of films (fig 3.7). Testing all the physical and chemical properties of all the films developed during such time frame was really challenging. However, we have come up with some interesting finding in this short time and that can be further continued in post-graduate studies which have relatively longer time.

6. Feasibility and future potentials

The oil leaking during the fish oil encapsulation was considerably lowered using modified alginate-carrageenan mixture. However, the stability of the beads was determined only after one week of incubation. And in further experiments the stability of the beads after longer time of incubation can be studied so that can add more commercial value of to the encapsulation process. Also, we have come up with the strong and thermally alginate-carrageenan mixed biofilms. They could be the alternative to the plastic packaging material and could be alternative to the polythene bags that can be degraded. Also, the biocompatibility of the fish oil encapsulated beads is another study that can be done in future. How the beads react to the human intestine could be another valuable study that can be carried out if future based on this research.

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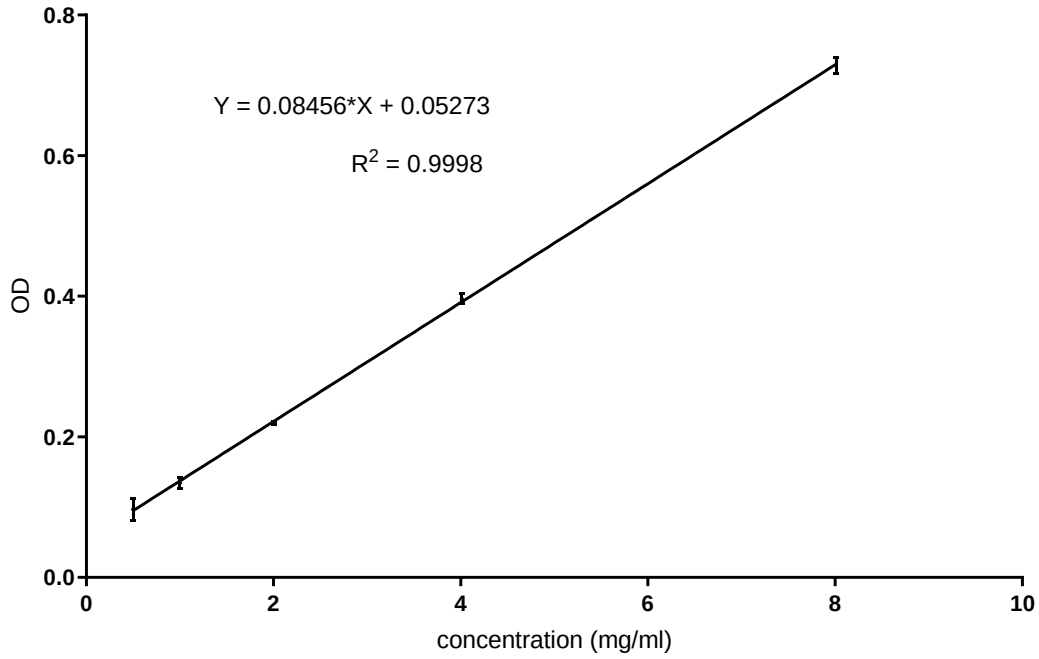
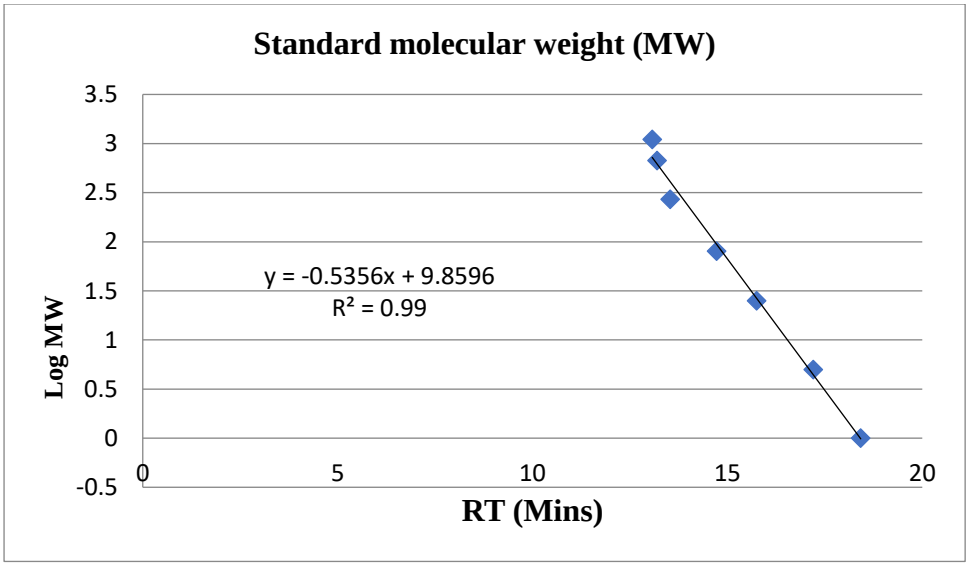
8. Appendix

Standard curve:



The standard curve was built adding fish oil in hexane and its serial dilution, and this curve was used to determine the concentration of the fish oil in gelling solution taking absorbance at 269 nm using UV-spectroscopy. It has shown the great linear relationship, and it was used for the determination of encapsulation efficiency and stability.

Standard curve for Molecular weight determination



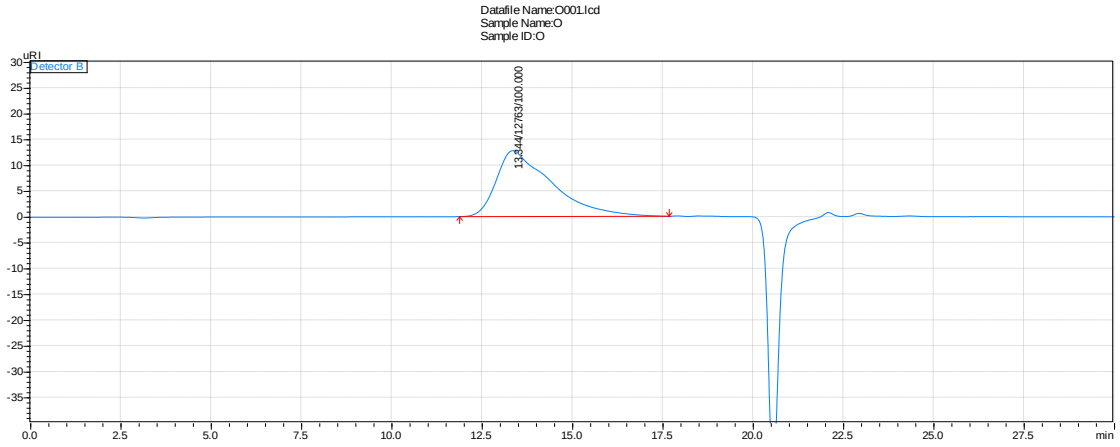
MW (kDa)	Log MW	RT (Mins)
1	0	18.421
5	0.69897	17.205
25	1.39794	15.752
80	1.90309	14.725
270	2.431364	13.529
670	2.826075	13.193
1100	3.041393	13.071

Mw=Molecular weight, RT= Retention time

Chromatogram of Molecular weight determination

The chromate gram shows a single peak for alginate molecular weight. It might show multiple peaks depending on the types of alginate and purity of alginate. The alginate used for the

experiment was of highest purity so most of the molecular weight showed a single peak**.** This peak shows the molecular weight of 478 kDa for oxidized alginate.



Peak#	Ret. Time	Name	Conc.	Area	%	Log MW	MW of Oxidized alginate*
1	13.344		100	1351222	100	2.679532	478.11459
Total			100	1351222		9.6451	

**Oxidation time 30 min using 30% hydrogen peroxide*